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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

New theoretical kinetic models for microbial biodegradation were developed based on growth and enzyme kinetics. These models were validated using individual bacterial cultures, and then they were applied to the kinetics of mineralization in waste water, fresh waters and soils. The observations on samples from these environments were in line with the theory. A conceptual model was also developed suggesting the existence of a threshold concentration below which growth-dependent mineralization will not occur, and the threshold

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level was verified with individual bacteria. Models were also devised and then tested for the simultaneous biodegradation of two organic chemicals present at low concentrations. Anomalies were found in the biodegradation of these low levels of organic compounds. Kinetics were tested for the cometabolism of toxic chemicals in fresh and waste waters. Reasons for the frequent failure of microbial inoculation to enhance biodegradation in natural environments were established. It was shown that the acclimation period prior to active biodegradation in waste water may be a result of protozoan grazing on the populations of mineralizing bacteria. Keywords:

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FINAL REPORT

TO ARMY RESEARCH OFFICE

BIODEGRADATION OF CHEMICALS AT TRACE

CONCENTRATIONS

GRANT NUMBER DAAG29-83-K-0068

May 30, 1986

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There have been extensive studies on the occurrence, kinetics, and products of microbial transformation of organic compounds. Many of these investigations were designed to serve as models to predict what will occur in natural water, soil, sewage, and other ecosystems. Researchers assumed that if a compound was mineralized, cometabolized, or resistant to microbial conversion at the levels normally used for biodegradation tests, it would be similarly mineralized, cometabolized, or resistant at the parts-per-billion level, or even lower levels, in nature. It also was assumed that the products would be the same regardless of substrate concentration and that the kinetics would be unchanged except that rates would decline in direct proportion to substrate concentration.

Our results show that erroneous conclusions may be reached from studies or routine tests done with organic chemicals at the levels often employed for predicting chemical fate in nature. These errors in extrapolation from high to low concentration may occur in routine evaluations of biodegradation, careful assessments of kinetics, or the establishment of products formed in water, soil, or sediments.

For the present purpose, high concentrations are not considered to be those toxic to common heterotrophic bacteria and fungi (that is, those species requiring organic compounds). Rather, the concentrations are in ranges not usually deemed to be inhibitory; for example they are in the range of 1-100 μg/ml of water or 1-100 μg/g of soil or sediment, on a dry-weight basis. Low concentrations, in contrast, are considered to be below the ranges cited; that is, in the parts-per-billion (ng/ml or ng/g) or parts-per-trillion (pg/ml or pg/g) range. The anomalies that occur at toxic chemical concentrations that we consider to be high lie outside the scope of this review because such levels are rarely encountered in nature.

Concern about the microbial metabolism of synthetic molecules at these low levels is of practical importance for several reasons. First, criteria and standards for water quality refer to maximum acceptable levels of many organic pollutants that are below 100 ng/ml. Second, numerous toxicants are harmful at levels in the parts-per-billion range, and risk assessments suggest that many others are probably injurious even in such trace amounts. Third, a substance may be nontoxic in the amounts that exist free in the water or outside the microbial cell in soil, but if the chemical is subject to bioconcentration, species at higher trophic levels may be harmed. Although the toxic chemical affecting the species at the higher trophic level is now at a high concentration within the organism, the chemical is not subject to microbial decomposition because it is within the tissues of the animal or plant and not free in water, soil, or sediments.

Several types of curves have been obtained in plots of the disappearance of organic molecules added to samples of natural environments or to pure cultures. On arithmetic axes, such substrate disappearance curves may be concave-up, as in first-order kinetics, or concave-down during nearly the entire period of decreasing substrate concentration, or the concentration of substrate or the appearance of product may appear to change linearly with time. In natural ecosystems, a variety of factors probably alter the shapes of substrate disappearance curves. The factors may include predation by protozoa, the time for induction of the active organisms, the accumulation of toxins produced by other microorganisms, depletion of inorganic nutrients or growth factors, the presence of other substrates which may repress utilization of the compound of interest, and binding of the compound to colloidal matter. The impacts or interactions of such potentially important factors may make it difficult to predict the kinetics of mineralization or disappearance of a particular substrate.

One approach to establish why substrate disappearance curves have so many different shapes is to seek an omnipresent minimum set of factors affecting the kinetics of biodegradation. In many instances, it is possible that the only factors or variables, at least for substrates that are mineralized, that need to be considered are the concentration of the compound and the abundance of active organisms. A study thus was designed to determine whether the variety of shapes of substrate disappearance curves could be modeled with only the variables of substrate concentration and population density and the parameters of classical Monod kinetics.

The rates of mineralization of $[^{14}C]$benzoate by an induced population of Pseudomonas sp. were measured at initial substrate concentrations ranging from 10 ng/ml to 100 μg/ml. Plots of the radioactivity remaining in the culture were fit by nonlinear regression to six kinetic models derived from the Monod equation. These models incorporate only the variables of substrate concentration and cell density. Plots of the mineralization kinetics in culture containing low, intermediate, and high initial substrate concentrations were well fit by first-order, integrated Monod, and logarithmic kinetics, respectively. Parameters such as maximum specific growth rate, half-saturation constant, and initial population density divided by yield agreed between cultures to within a factor of 3.4. Benzoate mineralization by microorganisms in acclimated sewage was shown to fit logistic (sigmoidal), Monod, and logarithmic kinetics when the compound was added at initial concentrations of 0.1, 1.0, and 10 μg/ml, respectively. The mineralization of 10 μg of benzoate per ml in sewage also followed logarithmic kinetics in the absence of protozoa. It is concluded that much of the diversity in shapes of mineralization curves is a result of the interactions of substrate concentration and population density.

We then developed a theoretical model for estimating threshold concentrations of organic substrates for bacterial growth. The model sets a physical limit on growth for bacteria in nutrient-poor environments. It is predicted that thresholds occur when maintenance energy requirements, in terms of substrate, equal the diffusive flux of substrate molecules to the bacterium. The model predicts either a negative growth rate or a diminishing cell size at concentrations at or below the threshold. We then applied the model to experimental studies of biodegradation of organic compounds.

Two general types of dual-substrate models can be distinguished, each of which requires a different approach to describe the kinetics of degradation of the test compound. The first class of models describing circumstances in which two compounds contribute substantially to the growth rate of the population has been considered above. Models of the second general type are for circumstances in which the substrate of interest is present at a very low concentration and therefore is not important in determining the growth rate of the active organisms. Growth of the active organisms in such cases would be governed almost entirely by the concentration of one or more alternative substrates. A study was thus designed to determine to what extent second substrates alter the kinetics of mineralization of low concentrations of organic compounds by pure cultures of bacteria.

We developed 12 models of kinetics to describe the metabolism of organic substrates that were not supporting bacterial growth. These models can be used to describe the biodegradation of organic compounds that are not supporting growth when the responsible populations are growing logistically, logarithmically, or linearly or are not increasing in numbers.

Nonlinear regression analysis was used to fit patterns of mineralization by two bacteria to these kinetic models. Pseudomonas acidovorans mineralized 1 ng of phenol per ml while growing exponentially at the expense of uncharacterized organic carbon in a synthetic medium. Phenol at a concentration of 1 ng/ml did not affect the growth of P. acidovorans. These data were best fit by the model that incorporates the equation for logarithmic growth and assumes a concentration of test substate well below its K_m value. In the absence of a second substrate, glucose at concentrations below those supporting growth was mineralized by Salmonella typhimurium in a manner best described by pseudo first-order kinetics. In the presence of different concentrations of arabinose, however, the kinetics of glucose mineralization by S. typhimurium reflected linear, logistic, or logarithmic growth of the population on arabinose. We conclude that the kinetics of mineralization of organic compounds at concentrations too low to support growth are best described either by the first-order model or by models that incorporate expressions for the kinetics of growth of the metabolizing population on other substrates. When growth is at the expense of other substrates, the kinetics observed reflect such growth, as well as the concentration of the substrate of interest.

In view of the availability of these new models, a study was initiated to determine their applicability to the kinetics of mineralization in lake water. For this purpose, two environmental pollutants, phenol and p-nitrophenol, were selected as test compounds. A wide range of concentrations was used, and samples of lake water were collected at several times of year.

The kinetics of mineralization of phenol and p-nitrophenol in lake water was determined at concentrations ranging from 200 pg to 5 ʁg/ml. The mineralization data were fit by nonlinear regression to equations for 14

kinetic models that describe patterns of biodegradation by nongrowing cells or by microorganisms growing on either the test chemical or other organic substrates. The kinetics of mineralization of phenol in water samples collected in July was best described by first-order models at 0.5 ng of phenol per ml; by Monod-without-growth, logistic, and logarithmic models at 1.0 ng, 2.0 ng, and 5.0 ng to $1.0 \mu g/ml$, respectively, if it is assumed that the mineralizing population uses phenol as the sole carbon source for growth; by models that assume the phenol-mineralizing population ar not growing or are growing logarithmically or logistically on uncharacterized carbon compounds but are metabolizing the phenol when present at levels below and above the K_m value, respectively, for that compound; and by a logarithmic model at 5.0 μg/ml. Under the test conditions, usually less than 10% of the phenol C that was metabolized was incorporated into microbial cells or retained by other particulate material in the water at substrate concentration of 10 ng/ml or less, and the percentage increased at higher substrate concentrations. The mineralization of 2.0 ng of phenol per ml in water samples collected at other times of year was best described by logistic or logarithmic models if the mineralizing bacteria were assumed to be growing on phenol, by a first order model, or by a model assuming logarithmic growth of the phenol mineralizers on other organic compounds in the water. If the lake water was incubated for 12 h before phenol was added, mineralization was zero-order. Removal of particles from lake water sampled after heavy run-off from land resulted in a change in kinetics of phenol mineralization. The patterns of mineralization of 0.5 ng to 1.0 μg of p-nitrophenol per ml were best fit by the Monod-with-growth model or by the logistic model if it is assumed that p-nitrophenol was the carbon source for growth, or by models that assume logarithmic or logistic growth on uncharacterized organic matter in the water.

Little attention has been given to the biodegradation of synthetic chemicals present at very low concentrations in soil. Low concentrations of pollutants are common in soil, originating from waste-disposal sites, pesticide application, fall-out from industrial stacks, or other sources. We propose above several kinetic models for the influence of substrate concentration on the kinetics of biodegradation, and we then used these models for assessing biodegradation kinetics of pure cultures of bacteria. The applicability of information on biodegradation kinetics measured in pure cultures to soil had yet to be demonstrated. In soil, the presence of barriers to diffusion, a large microbial community, low levels of available carbon sources, and different types of microorganisms may result in kinetics of biodegradation that are different from those observed in other environments or in pure cultures. Hence, an investigation was conducted to determine the kinetics of mineralization of low concentrations of several organic compounds in soil. Nonlinear regression techniques were used to fit the patterns of mineralization with models of kinetics developed for pure cultures and soil and with a new model proposed for the kinetics of biodegradation in soil.

The kinetics of mineralization of ^{14}C-labeled phenol and aniline was measured at initial concentrations ranging from 0.32 to 5000 ng and 0.30 ng to 500 $\mu g/g$ of soil, respectively. Mineralization of phenol at concentrations less than or equal to 32 ng/g of soil and of aniline at all concentrations began immediately, and the curves for the evolution of labeled CO_2 were biphasic. The patterns of mineralization of 4.0 ng of 2,4-dichlorophenol per g of soil and 20 ng of nitrilotriacetic acid per g of soil were similar to the patterns for phenol and aniline. The patterns of mineralization of 1.0 to 100 ng of p-nitrophenol and 6.0 ng of benzylamine per g

of soil were also biphasic but after a short apparent lag period. The curves of CO_2 evolution from higher concentrations of phenol and p-nitrophenol had increasing apparent lag phases and were S-shaped or linear. Cumulative plots of the percent of substrate converted to CO_2 were fit by nonlinear regression to first-order, integrated Monod, logistic, logarithmic, zero-order, three-half-order, and two-compartment models. None of the models of the Monod family provided the curve of best fit to any of the patterns of mineralization. The linear growth form of the three-half-order model provided the best fit for the mineralization of p-nitrophenol, with the exception of the lowest concentrations, and of benzylamine. The two-compartment model provided the best fit for the mineralization of concentrations of phenol below 100 ng/g, of several concentrations of aniline, and of nitrilotriacetic acid. Based on these data, we conclude that models derived from the Monod equation, including the first-order model, do not adequately describe the kinetics of mineralization of low concentrations of chemicals added to soil.

A study was initiated to determine whether it is possible to obtain nonlinear estimates of the parameters of Monod kinetics which describe the patterns of mineralization of organic substrates in cultures containing different cell densities and different concentrations of substrate. The kinetics of mineralization of a wide range of concentrations of benzoate, glucose, and benzylamine by Pseudomonas sp., Salmonella typhimurium, and microorganisms in acclimated sewage was studied. The treatment of initial substrate concentration and population density as independent variables in nonlinear regression analysis permitted the estimation of a single value for each of the parameters of Monod kinetics that best described the mineralization of substrate at each concentration by the pure cultures and

the sewage microflora. One value for each of the parameters of Monod
kinetics was used for each of the three compounds to produce theoretical
curves which lay close to the observed data on mineralization. Statis-
tically significant differences existed in the values of the parameters of
Monod kinetics that best described mineralization in cultures differing
only in initial substrate concentration and cell density. However, for the
compounds tested, the variance left by analyses using one value for each
parameter of Monod kinetics was less than double the unexplained variance
left by individual analyses of the data from each treatment. Although
significant, this increase is small compared with the amount of variance
that could be explained using only one value for each parameter of Monod
kinetics.

In self-accelerating models of degradation, growth of cells active on a
primary substrate occurs exclusively at the expense of that substrate. In
three-half-order models, the growth of active cells is independent of the
concentration of the primary substrate. Both types of models can apply to
the degradation of some compounds in some environments. A study was
undertaken to determine whether one of these two broad types of kinetics
offered a better characterization of the degradation of organic compounds
in samples of natural environments. The patterns of microbial mineral-
ization of 0.3 to 30 ng of glucose, benzoate, and phenol per ml of sewage
collected in late fall and winter were analyzed with the integrated Monod
equation and a model in which growth of active organisms occurs at the
expense of organic compounds other than the test substrate. Either model
could be closely fit by nonlinear regression to the data from individual
tests with one concentration of substrate added to one dilution of sewage.
However, neither model accounted satisfactorily for differences in patterns
of mineralization resulting from differences in substrate concentration and

cell density between different tests. It is suggested that both the added substrates and other organics present in sewage contributed to the growth of the active organisms. The mineralization of glucose in sewage collected in summer was better described by a two-compartment model than by any other model tested.

Studies were also conducted on the effects of second substrates on the biodegradation of low concentrations of organic compounds. Pseudomonas acidovorans and Pseudomonas sp. strain ANL but not Salmonella typhimurium grew in an inorganic salts solution. The growth of P. acidovorans in this solution was not enhanced by the addition of 2.0 μg of phenol per liter, but the phenol was mineralized. Mineralization of 2.0 μg phenol per liter by P. acidovorans was delayed 16 h by 70 μg of acetate per liter, and the delay was lengthened by increasing acetate concentrations, whereas phenol and acetate were utilized simultaneously at concentrations of 2.0 and 13 μg/liter, respectively. Growth of Pseudomonas sp. in the inorganic salts solution was not affected by the addition of 3.0 μg each of glucose and aniline per liter, nor was mineralization of the two compounds detected during the initial period of growth. However, mineralization of both substrates by this organism occurred simultaneously during the latter phases of growth and after growth had ended at the expense of the uncharacterized dissolved organic compounds in the salts solution. In contrast, when Pseudomonas sp. was grown in the salts solution supplemented with 300 μg each of glucose and aniline, the sugar was mineralized first, and aniline was mineralized only after much of the glucose carbon was converted to CO_2. S. typhimurium failed to multiply in the salts solution with 1.0 μg of glucose per liter. It grew slightly but mineralized little of the sugar at 5.0 μg/liter, but its population density rose at 10 μg of glucose per liter or higher. The hexose could be mineralized at 0.5 μg/liter, however,

if the solution contained 5.0 mg of arabinose per liter. In solutions with this arabinose concentration and glucose levels too low to support growth, the percentage of glucose carbon incorporated into S. typhimurium cells was the same as when the bacterium was grown in solutions with high concentrations of glucose alone. When glucose was the only carbon source for S. typhimurium, the percentage of the glucose carbon assimilated and mineralized progressively declined as the sugar concentration was reduced to levels approaching the threshold for growth. These results indicate that second substrates and uncharacterized dissolved organic carbon may play an important role in controlling the rate and extent of biodegradation of organic compounds at low concentrations.

During the course of our studies of the biodegradation of low concentrations of synthetic compounds in fresh water and sewage, we observed patterns of mineralization that were frequently unexpected based on investigations of higher concentrations of the same or related chemicals. These presumably anomalous findings were then evaluated since tests of biodegradation of organic compounds at concentrations not typical of natural ecosystems may lead to erroneous extrapolations about the transformations that occur in natural habitats. The rates of mineralization of nitrilotriacetic acid (NTA), 2,4-dichlorophenoxyacetic acid (2,4-D), p-nitrophenol, aniline, and isopropyl N-phenylcarbamate (IPC) at one or more concentrations ranging from 100 pg/ml to 1.0 μg/ml were proportional to chemical concentrations in samples of three lakes. The rates at 100 pg of NTA, 2,4-D, p-nitrophenol, and aniline per ml in samples of one or more lakes were less than predicted, assuming the rates were linearly related to the concentration. Neither NTA nor 2,4-dichlorophenol at 2.0 ng/ml was mineralized in some lake waters, but higher levels of the two chemicals were converted to CO_2 in samples of the same waters. In samples from two

lakes, little or no mineralization of IPC or 2,4-D occurred at 1.0 μg/ml, but 10 ng/ml or lower levels of the herbicides were mineralized. The mineralization in sewage of 1.0 μg of NTA per ml was biphasic; about 20% of the substrate was mineralized in 20 h, and mineralization was only reinitiated after a period of 130 h. The biphasic transformation was not a result of the accumulation of organic products, and it was still evident if protozoan activity was inhibited. NTA also underwent a biphasic mineralization in lake waters, and the biphasic pattern was not altered by additions of growth factors and inorganic nutrients. From 40 to 60% of the carbon of aniline added to lake water at levels of 100 pg/ml to 1.0 μg/ml was mineralized, but more than 90% of the carbon of NTA, 2,4-D, or p-nitrophenol added to lake water at 10 ng/ml or 1.0 μg/ml was mineralized. The microbial communities of lake water acclimated to degrade IPC and p-nitrophenol even below the minimal concentration at which bacteria can use single carbon sources for growth. IPC at 400 pg/ml and 1.0 ng/ml and 2,4-D at 100 pg/ml, 10 ng/ml, and 1.0 μg/ml were not mineralized in waters from all lakes.

Seven presumed anomalies were evident in this investigation of the biodegradation of low concentrations of organic compounds. (i) The rate of mineralization may be less than anticipated if it is assumed that the rates are linearly related to concentration. (ii) Chemicals mineralized at one concentration may not be converted to CO_2 at lower levels. (iii) Organic compounds may not be mineralized at low and presumably nontoxic levels in water, but they may be metabolized to CO_2 at still lower concentrations. (iv) Mineralization may not follow the commonly described kinetics but may proceed in a biphasic manner. (v). The extent of mineralization in samples from a single body of water may vary markedly. (vi) Microbial communities

may acclimate to mineralize a substrate even though the substrate concentration is below the threshold level to sustain growth. (vii) Compounds may be mineralized in some but not all waters. Given these putative anomalies, it seems likely that erroneous conclusions will be reached if knowledge from studies of chemicals at high concentrations is facilely extrapolated to environments in which the chemicals exist at low concentrations.

Microorganisms that cometabolize substrates convert them to organic products but do not obtain energy from the reaction or use carbon for biosynthesis. Although cometabolism may yield products which are toxic and persist in natural ecosystems, little information exists on the kinetics and factors affecting rates at chemical concentrations characteristic of those found in nature. Our studies on compounds that are mineralized have shown the importance of using concentrations characteristic of those in nature because the rates observed at the high concentrations commonly tested in the laboratory cannot always be used to predict the rates at the low concentration cha acteristic of many bodies of natural water. Moreover, a chemical may be mineralized at one concentration and cometabolized at another. Hence, we carried out a study to characterize the cometabolism of very low concentrations of three chemicals in sewage and eutrophic lake water. Factors affecting the rate of cometabolism of one of these compounds in samples from natural environments and in pure culture were also investigated.

Low concentrations of propachlor (2-chloro-N-isopropylacetanilide) and alachlor [2-chloro-2',6'-diethyl-N-(methoxymethyl)acetanilide] were not mineralized, cycloate (S-ethyl-N-ethylthiocyclohexanecarbamate) was slowly or not mineralized, and aniline and cyclohexylamine were readily mineralized in sewage and lake water. Propachlor, alachlor, and cycloate were

extensively metabolized, but the products were organic. Little conversion of propachlor and alachlor was evident in sterilized sewage or lake water. The cometabolism of propachlor was essentially linear with time in lake water and was well fit by zero-order kinetics in short periods and by first-order kinetics in longer periods in sewage. The rate of cometabolism in sewage was directly proportional to propachlor concentration at levels from 63 pg/ml to more than 100 ng/ml. Glucose but not aniline increased the yield of products formed during propachlor cometabolism in sewage. No microorganism able to use propachlor as sole source of carbon and energy was isolated, but bacteria isolated from sewage and lake water metabolized this chemical. During the metabolism of this herbicide by two of the bacteria, none of the carbon was assimilated. Our data indicate that cometabolism of these chemicals takes place at concentrations of synthetic compounds that commonly occur in natural waters.

Many synthetic organic compounds that are mineralizable persist in sewage, natural waters, and soils for some time before a population of microorganisms becomes sufficiently large or active to bring about their destruction. One way to enhance destruction of these chemicals is to inoculate the environment with microorganisms known to metabolize the chemicals readily. It is also possible that genetically engineered micro-organisms may be developed to bring about the rapid destruction of compounds that are slowly destroyed or that are not known to be mineralized in nature, and these newly constructed organisms would then be added to natural ecosystems after their growth in the laboratory or in large fermentors. Nevertheless, because abiotic stresses in natural environments are often different from those in the laboratory and because the introduced species may face intense competition, predation, or parasitism in nature, the inoculated organism may not bring about the desired biodegradation after its addition to sewage, natural waters, or soils.

The reasons for the inability of microorganisms added to natural environments or samples of these environments to bring about reactions they effect in culture are unknown. Because the use of naturally occurring or genetically engineered microorganisms represents a potentially promising means of destroying polluting chemicals in sewage, natural waters, or soils, a study was initiated to determine some of the reasons for the failure of microbial inocula to bring about degradative reactions in nature that they can catalyze in culture.

Pseudomonas strains capable of mineralizing 2,4-dichlorophenol (DCP) and p-nitrophenol (PNP) in culture media were isolated from soil. One DCP-metabolizing strain mineralized 1.0 and 10 μg of DCP but not 2.0 to 300 ng/ml in culture. When added to lake water containing 10 μg of DCP per ml, the bacterium did not mineralize the compound, and only after 6 days did it cause the degradation of 1.0 μg of DCP per ml. The organism did not grow or metabolize DCP when inoculated into sterile lake water, but it multiplied in sterile lake water amended with glucose or with DCP and supplemental nutrients. Its population density declined and DCP was not mineralized when the pseudomonad was added to nonsterile sewage, but the bacterium grew in sterile DCP-amended sewage, although not causing appreciable mineralization of the test compound. Addition of the bacterium to nonsterile soil did not result in the mineralization of 10 μg of DCP per g, although mineralization was evident if the inoculum was added to sterile soil. A second DCP-utilizing pseudomonod failed to mineralize DCP when added to the surface of sterile soil, although activity was evident if the inoculum, was mixed with the soil. A pseudomonad able to mineralize 5.0 μg of PNP per ml in culture did not mineralize the compound in sterile or nonsterile lake water. The bacterium destroyed PNP in sterile sewage and enhanced PNP mineralization in nonsterile sewage. When added to the

surface of sterile soil, the bacterium mineralized little of the PNP present at 5.0 μg/g, but it was active if mixed well with the sterile soil. These data suggest that microorganisms able to degrade organic pollutants in culture sometimes may fail to function when inoculated into natural environments because the concentration in nature may be too low to support growth or because the organisms may be susceptible to toxins or predators in the environments, may use other organic compounds in preference to the pollutant, or may be unable to move through soil to sites containing the chemical.

Studies of the efficiency of biomass production at the expense of energy and carbon source, i.e., the yield coefficient, are important for the analysis of both the kinetics of microbial processes in culture and the conversions of substrates in natural ecosystems to biomass, organic products, and CO_2. Large differences exist among values reported for the yield coefficient, however, and these variations make difficult the analysis of processes in culture and of the conversions in natural environments. Hence, a study was designed to determine the yield coefficients of a heterogeneous bacterial mixture, with special reference to the variable of cell density and substate concentration. Particular attention was given to determining the yield coefficients at trace concentrations of the organic substrate because low levels of numerous synthetic compounds, for example, less than 100 ng/ml, characterize the concentrations of pollutants in many fresh, estuarine, and marine waters. Measurements were made of the yield coefficient during the aerobic metabolism of glucose by a heterogeneous bacterial mixture. Expressed in terms of carbon, the coefficient was approximately 0.48. The value did not vary with initial bacterial densities ranging from 0.4 pg to 40 μg of cell carbon per ml and with glucose concentrations ranging from 43 pg to 100 μg of carbon per ml. Under all

these circumstances, about 44% of the glucose carbon was converted to CO_2 and 7.4% was excreted as organic products.

Research was conducted on the mechanisms responsible for the frequently long acclimation period prior to the biodegradation of low concentrations of pollutants. The mineralization of 2 ppb of PNP in sewage (as measured by counting the amount of radioactive CO_2 that was evolved) was detected on the second day of incubation. This suggests that the appearance of a mutant able to mineralize the PNP is not a likely mechanism for the delay in mineralization. To examine the role of protozoa in determining the length of the acclimation period in sewage, their numbers as a function of time were determined. Their population density in sewage amended with eucaryotic inhibitors declined to less than 100 per ml in 1 day. The total number rose to approximately 10^5 per ml on day 2 and remained at this density for 8 days. Most of these protozoa were of one type, a large ciliate, and this ciliate was shown to be resistant to the antibiotics. Counts of protozoa in sewage not receiving the inhibitors increased from less than 10^3 to greater than 10^4 in 1 day, then declined and leveled off at about 10^4 per ml.

To determine the population dynamics of the organisms biodegrading 2 ppb of PNP, fresh sewage was incubated with no amendments, with PNP, and with PNP and eucaryotic inhibitors. After a 1-day lag period, the number of PNP-degraders increased exponentially from approximately 100 to about 5 x 10^4 per ml after 8 days. The presence of PNP had no significant effect on the rate of increase or the final number of PNP-mineralizing organisms. However, in the presence of eucaryotic inhibitors, the number of PNP-degraders inc.eased rapidly after 1 day, from approximately 100 to greater

than 1000 per ml. The rapid increase of PNP-mineralizing organisms in sewage in which the protozoa were suppressed suggests that protozoan grazing is responsible for the acclimation prior to mineralization of PNP.

Eucaryotic inhibitors (cycloheximide and nystatin) reduced the length of the acclimation period by 10 days prior to the mineralization of 2, 10, and 100 ng of 2,4-D per ml and also reduced the extent of mineralization form 90 to 10%. The inhibitors reduced by 5 days the length of the acclimation period in sewage prior to the mineralization of 10 or 100 ng of 2,4-dichlorophenol per ml and reduced the extent of mineralization from 70 to 30%. These results confirm our earlier data with PNP that grazing by protozoa determines the length of the acclimation period prior to biodegradation in waste waters.

To determine the effects of an alternative prey on the acclimation period, a ciliated protozoan (Tetrahymena), Enterobacter sp., and an isolate capable of mineralizing PNP were added in various combinations to media containing 100 ng of PNP per ml. The presence of 10^8 Enterobacter cells per ml had no effect on PNP mineralization by the isolate (10^4 cells/per ml). When Tetrahymena, Enterobacter, and the isolate were present together at these cell densities, the length of the acclimation period was increased. Final cell densities of the isolate were reduced by the protozoa only in the presence of Enterobacter. Thus, in the presence of high densities of an alternative prey, protozoa will graze the low densities of a bacterium that is initiating biodegradation.

The presence of toxins, the need for alternative organic substrates, and protozoan grazing were apparently not responsible for the acclimation prior to PNP mineralization in lakewater. However, 10 µg of glucose per ml

increased the acclimation period prior to mineralization of 200 ng of PNP per ml. Addition of 10 nM phosphate increased the rate of mineralization of 10 ng of caprolactam per ml, and additions of 0.01-100 mM phosphate caused a significant shortening in the acclimation period prior to the mineralization of 2-2000 ng PNP per ml. The effect of phosphate was not a result of binding or precipitation of inhibitors.

Groundwater samples from different locations were tested for their ability to biodegrade various test compounds. Phenol at 100 ng/ml was mineralized in 1 day, and PNP at concentrations ranging from 1.0 to 1000 ng/ml was mineralized in 60 to 120 days. In groundwater naturally contaminated with various volatile organic compounds, no mineralization of phenol at 0.5-1000 ng/ml was observed in 28 days, although phenol at 0.1 ng/ml was mineralized in 28 days, approximately 10% of carbaryl at 2 ng/ml was mineralized in 28 days, but not mineralization of 100 ng/ml PNP, 2,4-D, and IPC was evident in 28 days. In groundwater obtained from a noncontaminated site close to the contaminated groundwater, PNP at 20 and 200 ng/ml was mineralized in 6 days, and phenol at 0.1, 0.5, 1.0, 5.0, 10, 100, and 1000 ng/ml was mineralized within 48 h.

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